INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(54) Title: SUBMICRON EMULSIONS AS VACCINE ADJUVANTS

(57) Abstract

A vaccine adjuvant composition of an oil-in-water submicron emulsion that has about 0.5 to 50 % of a first component of an oil, about 0.1 to 10 % of a second component of an emulsifier, about 0.05 to 5 % of a nonionic surfactant, about 0.00001 to 1 % of an immunogen, and an aqueous continuous phase. This submicron emulsion has a mean droplet size in the range of between about 0.03 and 0.5 μm, and preferably 0.05 and 0.2 μm.

SUBMICRON EMULSIONS AS VACCINE ADJUVANTS

FIELD OF THE INVENTION

This invention relates to the use of oil-in-water
submicron emulsions as vaccine adjuvants for enhancing
the immunogenicity and improvement of the immune response
of antigens and to methods and compositions for preparing
them.

BACKGROUND OF THE INVENTION

In the past, the risks of whole-pathogen vaccines
and limited supplies of useful antigens posed barriers to
development of practical vaccines. Today, the tremendous
advances of genetic engineering and the ability to obtain
many synthetic recombinant protein antigens derived from
parasites, viruses, and bacteria has revolutionized the
development of new generation vaccines.

Although the new, small synthetic antigens offer
advantages in the selection of antigenic epitopes and
safety, a general drawback of small antigens is poor
immunogenicity, resulting in low antibody titers and the
need for repeated immunizations. This lack of
immunogenicity has created an acute need to identify
pharmaceutically acceptable delivery systems or adjuvants
for these new antigens.

To improve the immune response usually antigens are
mixed with adjuvant substances that stimulate
immunogenicity. Immunological adjuvants have generally
been divided into two basic types: aluminum salts and oil
emulsions.

Aluminum phosphate and hydroxide (alum) have a long
history of use as adjuvants. They are the only ones
recognized as safe for this use by the Food and Drug
Administration. Antibody levels against antigens in
alum-based vaccines are clearly, although moderately,
elevated above those obtained with the corresponding

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aqueous vaccine. However, aluminum compounds have not always enhanced the immunogenicity of vaccines, and the problem of inconsistent antibody production has been frequently cited. Occasional production of sterile

5 abscesses and persistent nodules were also reported with alum-adjuvanted vaccines. Regarding long term side effects, researchers have suggested a link between aluminum and diseases of the brain, including Alzheimer's disease (Edelman, R.: Vaccine adjuvants. Rev. Inf. Dis.

10 1980; 2:370-383).

The development of emulsified oil adjuvants emerged historically from the studies of J. Freund who observed a remarkable increase in both the antibody and delayed hypersensitivity response to killed mycobacteria if the

15 organisms were incorporated in paraffin oil. There are two types of Freund's mineral-oil adjuvants: Incomplete Freund's Adjuvant (IFA), consisting of an approximately 50:50 water-in-oil emulsion, and complete Freund's adjuvant (CFA), a similar preparation with inclusion of

20 killed mycobacteria. The powerful antibody-stimulating effect of CFA has not been surpassed by any other adjuvant. However, because of severe pain, abscess formation, fever and granulomatous inflammation, CFA can be used only for experimental purposes and not in human

25 or veterinary vaccines. The toxic reactions reported using mineral oil-adjuvanted vaccines were attributed to impurities in Arlacel A (principally mannide monooleate), the emulsifying agent used in the preparations.

The use of IFA in humans has been limited to those

30 clinical situations in which aqueous vaccines are relatively impotent and aluminum compounds have not provided enough adjuvant activity. J. Salk made practical the use of IFA in human vaccines by using a highly refined mineral oil and a purified Arlacel A

35 emulsifier free of toxic substances injected intramuscularly in thousands of recipients. However,

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occasional failure of IFA vaccines reported in humans, and the discovery that Arlacel A was carcinogenic in mice, despite the absence of increased tumor formation in humans, has restricted the use of IFA vaccine
5 formulations.

Since CFA was the first successful adjuvant, most investigators followed the example of CFA in assuming that substitutes for each of the three components, viz. oil, emulsifier and immunostimulant, are necessary for
10 formulating a successful adjuvant.

U.S. Patents 4,772,466 and 4,606,918 disclose methods for enhancing the immunogenicity of an antigen by emulsifying it with a polyoxypropylene-polyoxyethylene block polymer, a glycol ether-based surfactant, a
15 metabolizable non-toxic oil, and an immunopotentiating amount of an immunostimulating glycopeptide.

Pharmaceutical compositions comprising an oil-in-water micron size emulsion, refined detoxified endotoxin, cell wall skeleton and trehalose dimycolate have been
20 disclosed as vaccine adjuvants in U.S Patents 4,505,900 and 4,803,070.

International patent application (PCT) WO 90/14837 discloses adjuvant composition comprising a metabolizable oil and emulsifying agent in the form of an
25 oil-in-water emulsion, where the antigen is added externally to the prepared emulsion (extrinsic formulation). All the examples in the disclosure contained the immunostimulating agent, MTP-PE, a lipophilic muramyl peptide derivative.
30

SUMMARY OF INVENTION

The present invention provides vaccine adjuvant compositions in the form of an emulsion of a plurality of submicron oil-in-water droplets having a particle size in
35 the range of between about 30 nm to about 500 nm to effect enhanced immunogenicity of antigens incorporated

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intrinsically or extrinsically into the droplets. These
droplets or particles form a submicron emulsion ("SME")
for use as the vaccine adjuvant.

In marked contrast to the aforementioned
5   disclosures, as will be described, the present invention
does not require use of any immunostimulatory
mycobacteria or muramyl peptide-like additives for its
submicron emulsion to be effective. Moreover, as will be
seen, a preferred embodiment of the present invention
10 consists of intrinsically incorporating the antigen into
the emulsion at the time of formation of the emulsion;
this is in distinct contrast to mixing the antigen with
the emulsion after the emulsion has been independently
extrinsically formed. It will be appreciated that
15 intrinsic formulation will be effective even in
situations and conditions and species where extrinsic
formulation is not. In this regard as well, the present
invention is uniquely different and not at all implied by
the previously mentioned applications which indeed
20 teaches away from the present invention in stating that
it is sufficient to simply mix the antigen with the
extrinsically previously formed emulsion.

The vaccine compositions of this invention also do
not include any polyoxypropylene-polyoxyethylene block
25 polymer, trehalose dimycolate, or cell wall skeleton, as
are found in prior art compositions.

Another aspect of this invention is to provide
compositions and methods for the preparation of submicron
emulsions containing antigens, incorporated either
30 intrinsically (emulsified together with the oil and
surfactant) or extrinsically (added externally to a
prepared SME).

In some cases, the submicron emulsion of the present
invention can be administered in combination with other
35 adjuvant systems, such as proteosomes, as indicated in
the examples.

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The size, concentration and specific formulation of
SMEs may be varied to suit the particular antigen used.
Moreover, such adjuvant preparations may enhance both
humoral and cell-mediated immunity (CMI) as do Freund's
5   adjuvants. The SMEs here described have been developed
for human use and since the oily droplets of the
emulsions are of submicron size and contain no added
pyrogenic moieties such as mycobacteria or MDP
derivatives they have, unlike Freund's adjuvants, great
10 safety potential. They may be especially applicable to
antigens that are vaccine candidates to protect against
biologic toxins or infectious agents, which have natural
hydrophobic moieties as a component including
transmembrane viral, bacterial or parasite proteins,
15 membrane proteins such as proteosomes,
lipopolysaccharides, glycolipids such as gangliosides, or
a variety of proteins or peptides to which hydrophobic
anchors have been chemically or genetically added.
       Another aspect of the invention provides
20 compositions and methods to achieve mucosal immunity by
using an emulsion comprising a plurality of submicron
particles, a mucoadhesive macromolecule, immunogenic
peptide or antigen, and an aqueous continuous phase,
which induces mucosal immunity by achieving mucoadhesion
25 of the emulsion particles to mucosal surfaces. Mucous
surfaces suitable for application of the emulsions of the
present invention may include-ocular (corneal,
conjunctival), oral (buccal, sublingual), nasal, vaginal
and rectal routes of administration.
30   The emulsion particles have a hydrophobic core
comprising a lipid or lipid-like composition and are
stabilized with amphiphilic and/or non-ionic surfactants.
       A wide variety of immunogens, including both water-
soluble and water-insoluble peptides or polysaccharides,
35 may be accommodated in the present emulsions. The
hydrophobic core and surfactant provide a

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microenvironment which accommodates lipophilic immunogens
such as lipid A or lipopolysaccharides as well as
membrane-associated peptide antigen domains, while the
aqueous continuous phase accommodates water-soluble
5 peptide domains, or oligosaccharides.

The term "peptide" herein includes both
oligopeptides and proteins. To facilitate intestinal
uptake, the emulsions may be encapsulated in gelatin
capsules or otherwise enterocoated to prevent their
10 exposure to gastric fluids when the oral route of
administration is selected. Furthermore, the emulsions
may be lyophilized as disclosed previously in Pharmos
Corp. International Application Publication WO 93/15736
prior to their encapsulation in order to achieve added
15 stability of the antigen.

BRIEF DESCRIPTION OF THE DRAWING FIGURES

Figure 1 is a graph showing the size distribution of
Intrinsic-SME vaccine containing SEB-Toxoid F antigen.
20 Figures 2A and 2B show the immune response obtained
after parenteral immunization with formalinized SEB-
Toxoid (Toxoid F) at two different antigen doses, 10 μg
(A) and 50 μg (B), formulated in extrinsic-SME,
intrinsic-SME, alum, or free antigen.
25 Figure 3 shows the immunogenicity of SEB-Toxoid F
complexed to proteosomes as a free antigen or adjuvanted
with alum or extrinsic SME.

Figure 4 shows the anti gp160 IgG antibody rabbit
titers obtained after parenteral immunization with gp160
30 formulated in alum, proteosomes or SME adjuvants.

Figure 5 shows the specific anti-Alex 10 (V3 loop)
rabbit IgG titers obtained after parenteral immunization
with gp160 formulated in alum, proteosomes or SME
adjuvants.
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DETAILED DESCRIPTION OF THE INVENTION
         This invention is directed to pharmaceutical
compositions comprising submicron emulsions as vaccine
adjuvants, and to methods for preparing and using such
5 compositions.

Features of the Submicron Emulsion (SME) Particles
         The submicron emulsion vaccine adjuvants of the
present invention comprise an aqueous continuous phase
10 suspending a colloidal phase of submicron particles. The
particles have a weighted average diameter of about 30 to
500 nm, more preferably about 50 to 200 nm. In many
embodiments, the weighted average diameter be less than
460 nm, 400 nm, 300 nm, or 200 nm.
15   Usually the weighted average diameter will be
greater than 40 nm or 50 nm, and frequently is greater
than 70 nm. Often, the above-stated upper and lower
diameter ranges will include both the weighted average
and at least one standard deviation of particle diameter.
20   The emulsion particle comprises a hydrophobic core,
often including or even consisting essentially of a
metabolizable and non-toxic oil such as MCT (medium chain
triglycerides) oil of the type extensively used in
parenteral emulsions like INTRALIPID® or a vegetable oil.
25 Optionally, other hydrophobic lipids may be used,
including cholesterol or cholesteryl esters and fatty
acids. In many embodiments, the core of the particles
will be substantially free of protein other than the
antigen to be delivered, i.e. less than 1% (w/w), and in
30 most cases less than 0.1% of other protein.
         The emulsion usually further includes at least one
surfactant, which may be a natural biologically
compatible surfactant such as phospholipid (e.g.,
lecithin) or a pharmaceutically acceptable non-natural
35 surfactant such as TWEEN-80. The surfactant assists in

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maintaining particles within the desired size range and preventing their aggregation.

In many embodiments the emulsion may be formed and stabilized in the substantial absence of one or more
5   cosurfactants selected from the group consisting of an unhalogenated aliphatic C3-C6 alcohol, a free fatty acid, a mono- or di-glyceride, a polyglycerol fatty acid ester, or a lysophosphatidyl choline. One or all of the above-named cosurfactants may comprise less than 5%, commonly
10 less than 1%, and frequently less than 0.1% (w/w) relative to the weight of the hydrophobic core.

The emulsion also contains an immunogen, preferably of an antigen. The antigen may be hydrophilic, hydrophobic, or amphiphilic since the emulsion provides a
15 biphasic lipophilic-hydrophilic microenvironment.

The continuous phase of the emulsion is aqueous, and may contain salts, sugars, antioxidants, preservatives, microbicides, buffers, osmoticants, cryoprotectants, and other pharmaceutically useful additives or solutes.
20   Bioadhesive polymers, such as those currently used in pharmaceutical preparations, optionally may be added to the emulsion to further enhance the immunogenicity through mucous membranes achieving mucosal immunity.

The concentrations indicated by % in the following
25 description denote the concentration by weight of the component per 100 units volume of the entire composition. Also, all indicated concentrations should be understood as standing each by itself, and not cumulative. It should be appreciated by the artisan, however, that there
30 is some dependency between the concentrations of the components, e.g., higher concentrations of the oil will generally require higher concentrations of the emulsifier and surfactant.

The emulsion used in the vaccine compositions of the
35 present invention typically contain about 0.5 to 50% oil, about 0.1 to 10% emulsifier and about 0.05 to 5% of the

non-aqueous phase. The combined concentration of the oily and the amphiphilic phase increases viscosity of the composition. In order to obtain a non-viscous composition, the concentration of the non-aqueous phase
5   should generally not exceed about 25%.

Preferred concentrations of the components are as follows: about 1 to 20% oil, most preferably about 1 to 10% for a composition intended to be fluid, about 0.2 to 5% of the emulsifier, with about 0.2 to 5% for the
10 surfactant, with about 0.1 to 2% being particularly preferred.

The antigen is present in an amount of about 0.00001 to 1% by weight of the composition, preferably about 0.0001 to 0.5% and most preferably 0.001 to 0.1%.
15 Depending upon whether the antigen is hydrophilic or hydrophobic, it will be physically present in the oily phase at the oil-water interface, or the aqueous component. Also, the pH of these compositions should be in a range which is suitable for the stability of the
20 antigen.

The submicron emulsion adjuvant formulations of this invention differ from the emulsion adjuvant composition of Patent Application WO 90/14837 in the following features:

25   (i)   all the compositions described in the
            abovementioned application are prepared
            extrinsically, namely the antigens are added
            externally to the previously prepared emulsion by
            mixing, while in the present invention the antigen
30   can be added either extrinsically or more preferably
            intrinsically, together with all the emulsion
            components before emulsification and prior to the
            mixture of oil and water phases as detailed in the
            examples; and

35   (ii) all the examples in the abovementioned
            disclosure contain an immunopotentiating amount of
            an immune-stimulating glycopeptide of the type of

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muramyl peptides or their lipophilic derivatives,
such as MTP-PE, while in the present invention all
the SME adjuvant compositions are prepared in the
absence of any muramyl peptide immunostimulating
5   agent.

Composition of the Hydrophobic Core

A hydrophobic compound which is suitably non-toxic
may be used as a component of the core. Examples include
10   triglycerides, preferably of food grade purity or better,
which may be produced by synthesis or by isolation from
natural sources. Natural sources may include animal fat
or vegetable oil, e.g., soya oil, a source of long chain
triglycerides (LCT). Other triglycerides of interest are
15   composed predominantly of medium length fatty acids,
denoted medium chain triglycerides (MCT). A medium chain
triglyceride (MCT) oil, is a triglyceride in which the
carbohydrate chain has 8-12 carbons. Although MCT oil
can be considered as a component of vegetable oil, it is
20   separately identified herein because of its particular
utility as a preferred oil for use in the present
emulsions. In addition, MCT oil is available
commercially. Examples of such MCT oils include TCR
(trade name of Societe Industrielle des Oleagineuax,
25   France, for a mixture of triglycerides wherein about 95%
of the fatty acid chains have 8 or 10 carbons) and
MIGLYOL 812 (trade name of Dynamit Nobel, Sweden for a
mixed triester of glycerine and of caprylic and capric
acids). The fatty acid moieties of such triglycerides
30   may be unsaturated, monounsaturated or polyunsaturated;
mixtures of triglycerides having various fatty acid
moieties are acceptable. The core may comprise a single
hydrophobic compound or a mixture of compounds.
Examples of vegetable oils include soybean oil,
35   cotton seed oil, olive oil, sesame oil and castor oil.
Oily fatty acids, such as oleic acid and linoleic acid,

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fatty alcohols, such as oleyl alcohol, and fatty esters, such as sorbitan monooleate and sucrose mono-, di- or tripalmitate, can be used as the oil component, although these are not as preferred as the other oils mentioned
5   above.

Optionally, the core may contain cholesterol or cholesteryl esters. In many embodiments, cholesteryl esters or cholesterol comprise less than 10%, 5%, 1%, or even 0.1% (w/w) of the total hydrophobic components of
10 the core. Considerations in choice of core material include low toxicity and irritancy, biocompatibility, safety, metabolizability, stability and high loading capacity for antigens. Preferred hydrophobic core components have molecular weights below about 5,000 Da,
15 more preferably below about 2,000 Da, and most preferably below about 1,500 Da.

Composition of Surfactant Component

The amphiphilic phase comprises the emulsifiers and
20 surfactants. Preferred emulsifiers include a phospholipid compound or a mixture of phospholipids. Suitable components include lecithin; EPICURON 120 (Lucas Meyer, Germany) which is a mixture of about 70% of phosphatidylcholine, 12% phosphatidylethanolamine and
25 about 15% other phospholipids; OVOTHIN 160 (Lucas Meyer, Germany) which is a mixture comprising about 60% phosphatidylcholine, 18% phosphatidylethanolamine and 12% other phospholipids; a purified phospholipid mixture-LIPOID E-75 or LIPOID E-80 (Lipoid, Germany) which is a
30 phospholipid mixture comprising about 80% phosphatidylcholine, 8% phosphatidylethanolamine, 3.6% non-polar lipids and about 2% sphingomyelin. Purified egg yolk phospholipids, soybean oil phospholipids or other purified phospholipid mixtures are useful as this
35 component. This listing is representative and not

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limiting, as other phospholipid materials which are known to those skilled in the art can be used.

Some embodiments of the invention provide an improved bioadhesive emulsion comprising incorporation of
5 an amphiphilic and/or nonionic surfactant such as phosphatidylcholine, Tween, etc., together with a mucoadhesive polymer macromolecule as described below.

Particularly suitable emulsifiers include phospholipids, which are highly biocompatible.
10 Especially preferable phospholipids are phosphatidylcholines (lecithins), such as soy or egg lecithin. Other suitable phospholipids include phosphatidylglycerol, phosphatidylinositol, phosphatidylserine, phosphatidic acid, cardiolipin, and
15 phosphatidylethanolamine. The phospholipids may be isolated from natural sources or prepared by synthesis. Phospholipid surfactants are believed usually to form a single monolayer coating of the hydrophobic core.

The surfactant is believed in many embodiments to
20 interact with the bioadhesive polymer to form a hydrated polymer film coating associated with the surfactant at the stabilized lipid/water interface surrounding the particle core.

Preferred compositions contain a surfactant
25 component. The surfactant stabilizes the outer surface of the hydrophobic core component of the emulsion particles, thereby promoting a more uniform and manipulatable particle size. Usually the surfactant is present in a proportion of 0.01% to 5% (w/w) of the
30 emulsion, preferably 0.05% to 2% and most preferably 0.1 to 2%.

Typically, the weight percentage of surfactant relative to hydrophobic (oil or other lipid) component is from 0.2% to 50%, more preferably from 5% to 20%. Higher
35 ratios of surfactant to core lipid tend to promote smaller particle core diameters.

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Surfactants may be either natural compounds, such as phospholipids and cholates, or non-natural compounds such as: polysorbates, which are fatty acid esters of polyethoxylated sorbitol (TWEEN); polyethylene glycol
5 esters of fatty acids from sources such as castor oil (EMULFOR); polyethoxylated fatty acid, e.g., stearic acid (SIMULSOL M-53); NONIDET; polyethoxylated isooctylphenol/formaldehyde polymer (TYLOXAPOL); polyoxyethylene fatty alcohol ethers (BRIJ); polyoxy-
10 ethylene nonphenyl ethers (TRITON N); polyoxyethylene isooctylphenyl ethers (TRITON X). Mixtures of surfactant molecules, including mixtures of surfactants of different chemical types, are acceptable. Surfactants should be suitable for pharmaceutical administration and compatible
15 with the peptide to be delivered.

In certain embodiments, the emulsion may be limited in or substantially free of one or more cosurfactants selected from the group consisting of free fatty acids, mono- or diglycerides (fatty acid mono- or diesters of
20 glycerol), aliphatic C3-C6 monoalcohols (exclusive of, e.g., chlorobutanol or other haloalkyl alcohol preservative), polyglycerol fatty acid esters, or lysophosphatidyl choline. In many embodiments, the particular limited cosurfactant from the above group may
25 constitute less than 5%, usually less than 1%, often less than 0.1%, relative to the weight of hydrophobic core component. In some embodiments, one or more cosurfactants may be present.

30 **Continuous Aqueous Phase**

The aqueous component will be the continuous phase of the emulsion and may be water, saline or any other suitable aqueous solution which can yield an isotonic and pH controlled preparation.

35   In addition, the compositions of the invention may also comprise conventional additives such as

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preservatives, osmotic agents or pressure regulators and
antioxidants. Typical preservatives include Thimerosal,
chlorbutanol, and methyl, ethyl, propyl or butyl
parabens. Typical osmotic pressure regulators include
5 glycerol and mannitol, with glycerol being preferred.
The preferred oil phase antioxidant is α-tocopherol or α-
tocopherol succinate. The aqueous phase may also include
an antioxidant of a polyamine carboxylic acid such as
ethylene diamino tetraacetic acid, or a pharmaceutically
10 acceptable salt thereof.

Antigens

     Since the SME particles provide a hydrophilic-
lipophilic microenvironment, either water-soluble or
15 lipid-soluble immunogens can be incorporated in the SME
vaccines of the present invention. Examples of peptide
antigens are: hydrophilic natural or synthetic peptides
and proteins derived from bacteria, viruses and
parasites, such as the recombinant gp160 envelope protein
20 of the HIV virus; natural or synthetic glycoproteins
derived from parasites, bacteria or viruses such as the
native surface glycoprotein of *Leishmania* strain or
subunit vaccines containing part of the glycopeptides
alone or covalently conjugated to lipopeptides like
25 lauryl-cystein hydrophobic foot; protein toxoids such as
the Staphylococcus enterotoxin B toxoid, either
chemically or physically inactivated, non-toxic bacterial
surface structures (fimbrial adhesions) of *Eschesichia
coli* strains such as the Shiga-like Toxin B Subunit (SLT-
30 B) and AF-RL, a pilus adhesion which is a virulence
factor for RDEC-1 *E. coli* strain; outer membrane proteins
of *Neisseria meningitides*; Hepatitis B surface antigen;
native or synthetic malaria antigens derived from
different portions of *Plasmodium falciparum,* etc.
35   Examples of lipophilic or hydrophobic immunogens are
lipopolysaccharides (LPS), such as detoxified LPS

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obtained from E. coli (Sigma Chemical Co., St. Louis, USA); Lipid A, the terminal portion of LPS, such as the one isolated from Salmonella minnesota R595 from List Biological Laboratories (CA, USA).

5   In some embodiments, the emulsion particles will be free or substantially free of the above or other nonbioactive proteins, i.e. less than 5%, usually less than 1%, and frequently less than 0.1% (w/w) protein relative to other particle components.

10

Bioadhesive SME Vaccine Adjuvants

Submicron emulsion vaccine adjuvants of the present invention optionally may contain a bioadhesive macromolecule or polymer in an amount sufficient to

15   confer bioadhesive properties. The bioadhesive macromolecule enhances the delivery and attachment of antigens on or through the target mucous surface conferring mucosal immunity. The bioadhesive macromolecule may be selected from acidic non-naturally

20   occurring polymers, preferably having at least one acidic group per four repeating or monomeric subunit moieties, such as polyacrylic acid and/or polymethacrylic acid (e.g. CARBOPOL, CARBOMER), poly(methylvinyl ether/maleic anhydride) copolymer, and their mixtures and copolymers;

25   acidic synthetically modified natural polymers, such as carboxymethylcellulose (CMC); neutral synthetically modified natural polymers, such as (hydroxypropyl) methylcellulose; basic amine-bearing polymers such as chitosan; acidic polymers obtainable from natural

30   sources, such as alginic acid, hyaluronic acid, pectin, gum tragacanth, and karaya gum; and neutral non-naturally occurring polymers, such as polyvinylalcohol; or their mixtures.

The ionizable polymers may be present as free acids,

35   bases, or salts, usually in a final concentration of 0.01-0.5% (w/v).

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As used herein, a polymer of an indicated monomeric subunit contains at least 75%, preferably at least 90%, and up to 100% of the indicated type of monomer subunit; a copolymer of an indicated type of monomeric subunit
5   contains at least 10%, preferably at least 25% of that monomeric subunit.

A preferred bioadhesive macromolecule is the family of acrylic acid polymers and copolymers (e.g. CARBOPOL™). These contain the general structure:
10

$$-[-CH_2-CH(COOH)-]-n$$
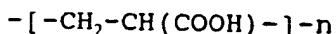
One preferred group of polymers of acrylic acid is commercially available under the tradename CARBOPOL.
15   CARBOPOL 934 is available in a pharmaceutical grade.

Preferred bioadhesive or mucoadhesive macromolecules have a molecular weight of at least 50 kDa, preferably at least 300 kDa, and most preferably at least 1,000 kDa. Favored polymeric ionizable mucoadhesive macromolecules
20   have not less than 2 mole percent acidic groups (e.g. $COOH$, SO_3H) or basic groups (NH_2, NRH, NR_2), relative to the number of monomeric units. More preferably, the acidic or basic groups constitute at least 5 mole percent, more preferably 25 or even 50, up to 100 mole %
25   relative to the number of monomeric units of the macromolecule.

Preferred macromolecules also are soluble in water throughout their relevant concentration range (0.01-0.5% w/v).
30

Methods of Preparation

A further embodiment of the invention relates to methods for preparation of submicron emulsion vaccine adjuvants intrinsically and extrinsically as extensively
35   detailed in the examples. In general, SME intrinsic formulations are prepared by emulsifying the antigen

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together with the SME components, while SME extrinsic
formulations are prepared by adding externally the
antigen to previously prepared plain SME.

5   **Dehydrated SME Adjuvants**

A further aspect of the invention provides
dehydrated emulsions, made by dehydrating the submicron
emulsion of the types described herein. Dehydrated
submicron emulsions may be stored for prolonged periods
10 with minimal degradation, then reconstituted with water
shortly before use. Residual water content in the
dehydrated emulsion is usually less than 5% (w/w),
commonly less than 2%, and often less than 1%.

Dehydration may be performed by standard methods,
15 such as drying under reduced pressure; when the emulsion
is frozen prior to dehydration, this low pressure
evaporation is known as lyophilization. Freezing may be
performed conveniently in a dry ice-acetone or ethyl
alcohol bath. The pressure reduction may be achieved
20 conveniently with a mechanical vacuum pump, usually
fitted with a liquid nitrogen cold trap to protect the
pump from contamination. Pressures in the low millitorr
range, e.g., 10-50 millitorr, are routinely achievable
but higher or lower pressures are sufficient.

25   A cryoprotectant or anticoalescent compound may be
added to the emulsion prior to dehydration to inhibit
flocculation and coalescence upon rehydration. The
cryoprotectant may be of any type known in the art,
including sugars and polysaccharides such as sucrose or
30 trehalose, and non-natural polymers such as
polyvinylpyrrolidone. Cryoprotectants are usually
present at less than 25%, commonly 10%, more commonly 5%,
4% (w/v) or less in the emulsion before lyophilization.

A preferred category of cryoprotectants is amino
35 acids and oligopeptides. Preferred amino acids include
valine, leucine, isoleucine, lysine, methionine,

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threonine, serine, arginine, alanine, glycine, histidine, proline, phenylalanine, taurine, and carnitine, although any of the other natural amino acids may also be present. Amino acids may be of either D or L configuration, or a
5   mixture; the natural L form is preferred. Amino acids may be present as their salts or esters, and as mixtures of amino acids or as pure species.

A particularly preferred amino acid is glycine, which may be present either in pure form or as a
10   component of a mixture, e.g. in an hydrolyzate of collagen or other glycine-rich protein.

Mixtures of oligopeptides, especially di- and tripeptides, are another preferred type of cryoprotectant. These may be prepared conveniently as
15   partial protein hydrolysates or enzymatic digests.

The cryoprotective amino acids or oligopeptides are generally present in the emulsion at a concentration of about 0.25 to 25% (w/w), preferably about 0.5 to 12% (w/w), more preferably about 1 to 10% (w/w) and commonly
20   3-6% (w/w).

Cryoprotectants and methods of making lyophilized submicron emulsions are taught in more detail in Pharmos Corp. PCT International Application Publication WO 93/15736 entitled "Dry Compositions for Preparing
25   Submicron Emulsions", the content of which is expressly incorporated herein by reference.

EXAMPLES

This invention is illustrated by the following non-
30   limiting examples:

Example 1:   Preparation of Intrinsic-gp160-SME vaccine
Antigen description and background. The urgency and high priority for developing an effective vaccine against the
35   human immunodeficiency virus (HIV) are fully recognized. The reasons for using subunits of the virus as the basis

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of an HIV vaccine are the perceived overwhelming
requirements for safety. Despite the high efficacy of
many live attenuated viral vaccines, the requirement for
product safety, especially in the case of retroviruses,
5   has favored the subunit approach to the extent that all
of the current candidate preparations in clinical
prophylactic trials are of this type, being mainly gp160,
the envelope protein of HIV, or part thereof. Studies
have shown that gp160 attaches the virus to the cell and
10   also facilitates the fusion of the cell and virus during
the early stages of infection.

The gp160 antigen used in this example was supplied by
MicroGeneSys Inc. This gp160 recombinant protein in
15   alum-adjuvanted vaccine formulation is currently under
evaluation in human clinical trials.

Preparation of oil phase
The oil phase was composed of MCT oil (2.0 g Mygliol 812,
20   Hulls, Germany), lecithin (0.4 g, Lipoid E-80, Germany),
and DL-α-tocopherol succinate (8.0 mg, Merck, Germany).
The lipids and oil were weighed in a 250-ml beaker and
mixed at room temperature using a magnetic stirrer during
2-4 hrs until a homogenous and almost clear solution was
25   obtained.

Preparation of water phase
Polysorbate 80 (1 % w/v, Montanox 80, DF, Seppic,
France), Glycerol (2.2% w/v, Merck, Germany), EDTA (0. 1
30   % w/v, Merck, Germany), and purified water (to 100% w/v)
were dissolved at room temperature in a 250-ml beaker by
gentle shaking using a magnetic stirrer plate until a
clear homogenous solution was obtained (about 15-20 min).
A total volume of 40 ml of water phase was prepared. A
35   vial containing 2.1 ml of gp160 recombinant protein
(MicroGeneSys, Inc., CT, USA) at a concentration of 0.25

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mg/ml in saline was added to the water phase and the
mixture was gently shaken for 5 min.

Mixing of oil and water phase
5 The oil phase was heated to 40°C and added to the beaker
containing the 40 ml of water phase. The mixture was
gently stirred for 10-15 min at room temperature.

Preparation of oil-in-water coarse emulsion
10 An oil-in-water emulsion containing the antigen was
prepared using the medium-sized disperser and
homogenizing unit Polytron PT3000 (Kinematics,
Switzerland) at 3,600 rpm for 30 sec. The resultant
micronsize emulsion was cooled at room temperature.
15

Sizing of emulsion to submicron range
The droplet size of the emulsion obtained after Polytron
step was lowered to the submicron (nanosize) range by
submitting the emulsion to high shear homogenization
20 using the Gaulin Microlab 70 High Pressure Homogenizer
(APV Gaulin, Germany) at 800 bar pressure. A total of 10
cycles were performed. The particle size distribution of
the resultant formulation was determined using an N4MD
Coulter Particle Size Analyzer (Coulter Electronics,
25 England). The differential weight % mode of the
instrument indicated the existence of homogeneous
population of SME droplets with a mean particle size
distribution of 43±35 nm. The estimated final gp160
concentration in the formulation was 56 μg/ml.
30

Example 2:   **Preparation of Intrinsic-SME vaccine**
                     containing gp160 complexed to proteosomes

Proteosomes are meningococcal outer membrane protein
35 preparations purified from *Neisseria meningitidis* by
detergent extraction and ammonium sulphate precipitation.

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They naturally form 20-100 nm diameter hydrophobic
membraneous vesicles. Antigens are non-covalently
complexed to proteosomes via hydrophobic interactions by
mixing the antigen and proteosomes in the presence of
5   detergent and then removing the detergent over a
prescribed period of time, permitting hydrophobic
interactions to occur in the system.

Proteosomes have previously been shown to enhance the
10   parenteral immunogenicity of peptides, gangliosides,
lipopolysaccharides and proteins hydrophobically
complexed to them (Lowell, G.H., L.F. Smith, R.C. Seid
and W.D. Zollinger, J. Exp. Med. 167: 658-663, 1988).
(Lowell, G.H., W.R. Ballou, L.F. Smith, R.A. Wirtz, W.D.
15   Zollinger and W.T. Hockmeyer. Science 240: 800-802,
1988; Lowell, G.H. 1990. In: New Generation Vaccines.
G.C. Woodrow and M.M. Levine (eds.), Marcel Dekker, Inc.,
New York, p. 141-160). and have been shown to be safe for
human use in vaccine trials involving tens of thousands
20   of humans in the development of anti-meningococcal
vaccines (Zollinger, W.D. New and Improved Vaccines
Against Meningococcal Disease. In: New Generation
Vaccines, G.C. Woodrow and M.M. Levine (eds.), Marcel
Dekker, Inc., New York, p. 325-348). Furthermore,
25   proteosomes confer mucosal immunogenicity upon non-
immunogenic antigens when administered orally or
intranasally. Such intranasal or oral proteosome
vaccines induce up to 100% protection against lethal
pneumonia or keratoconjunctivitis in experimental murine
30   models of shigellosis (Orr, N., G. Robin, D. Cohen, R.
Arnon and G. Lowell. 1993. Immunogenicity and efficacy
of oral or intranasal *Shigella flexneri* 2a and *Shigella
sonnei* proteosome-lipopolysaccharide vaccines in animal
models. Infect. Immun. 61: 2390-2395).
35

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Oil and aqueous phases were prepared as described in
Example 1. A vial containing 2.5 mg of gp160 non-
covalently complexed to proteosomes and suspended in
saline was added to the water phase (40 ml total volume)
5   and the mixture was gently shaken for 5 min. The
subsequent steps involved in the preparation of the SME,
i.e. mixing of oil and water phases, homogenization and
sizing to submicron range were carried out as described
in Example 1. The particle size volume % distribution of
10 the resultant formulation showed a mean droplet size of
38±41 nm. The estimated final gp160 concentration in the
formulation was 46 μg/ml.

Example 3:   Preparation of Extrinsic-gp160-SME vaccine
15

Extrinsic formulations of gp160 in SME were performed by
preparing plain SME as described in Example 1, but in the
absence of the antigen and adding externally the aqueous
solution containing the gp160 to the plain SME by gently
20 shaking for 15 min at room temperature. A total volume
of 2.3 mi of plain SME (average droplet size of 50±36 nm)
were mixed with 2.1 ml solution of gp160 in saline
containing 1.1 mg protein to give a final gp160
concentration of 0.25 mg/ml.
25

Example 4:   Preparation of Extrinsic-SME vaccine
containing gp160 complexed to proteosomes

Extrinsic formulation of gp160 conjugated to proteosomes
30 in SME were performed by preparing plain SME as described
in Example 1, but in the absence of the antigen and
adding externally an aqueous dispersion of the gp160-
conjugated to proteosomes to the plain SME by gently
shaking for 15 min at room temperature. A total volume
35 of 5.4 ml of plain SME (average droplet size of 50±36 nm)
were mixed with 7.0 ml saline containing 3.1 mg of gpl 60

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complexed to proteosomes to give a final gp160
concentration of 0.25 mg/ml.

Example 5:   Preparation of Intrinsic-SME vaccine
                  containing Staphylococcus Enterotoxin B
                  Toxoid-F

Antigen description and background: Staphylococcal
enterotoxin B (SEB) is a potent toxin that causes food
borne illness among civilians and military personnel
stationed around the world and is identified as a lethal
offensive military threat that endangers both military
and civilian populations through aerosolization.

SEB infection in civilian populations is related to
staphylococcal food poisoning by SEB and related toxins:
also contributes to death from staphylococcal sepsis
following overwhelming staph infection. It also causes
staph scalded skin syndrome in kids - i.e. morbidity and
mortality from staphylococcal infections (P. Marrack and
J. Kappler, Science, vol. 248, pp. 705-711.)

Due to the similarity to the human response both in
sensitivity and clinical signs and the lack of an
established model for lethality to SEB delivered via the
respiratory route in lower animal species, non-human
primates have been the primary animal model for
development of vaccines to protect against respiratory
challenge with SEB. Early work indicated that monkeys
develop decreased sensitivity to repeated mucosal
administration of the toxin. This suggested that
protection to SEB exposure might be provided by toxoid
immunization. Studies in rhesus monkeys and other
animals indicated that oral immunization with
formalinized toxoid was ineffective against parenteral
challenge whereas parenteral immunization with
formalinized SEB toxic induced serum antibodies that
recognized native SEB (Bergdoll, M.S. Enterotoxins. pp.

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559-598 In: Staphylococci and Staphylococcal Infections,
eds. C.S.F. Easmon and C. Adlam, Academic Press, London,
1983). In the latter studies, however, several
parenterally immunized monkeys that acquired anti-SEB
5   antibodies had severe immediate-type hypersensitivity
reactions when challenged with SEB toxin. These adverse
reactions suggested that the formalinized SEB toxoid
alone was not a candidate for parenteral vaccine
development. Additionally, as the military threat would
10   be by aerosolization, it was determined that studies on
protection provided by serum IgG to respiratory challenge
as well as protective effects provided by anti-SEB
secretory IgA in the respiratory tract were required.

15   Recently, two identical lots of formalinized SEB toxoid
were made at Walter Reed Army Institute of Research,
Washington DC (WRAIR) following previously described
specifications (Kaminski, R., S. Grate, E. Aboud-Pirak,
C. Hooper, T. Levin, I. Weiss, S. Amselem, R. Arnon and
20   G. Lowell, 1993). Comparison of induction of respiratory
IgA and serum lg by intranasal and parenteral proteosome
vaccines formulated with staphylococcal enterotoxin B
toxoids or ricin peptides. In Proceedings of 1993
Medical Defense Bioscience Review, Baltimore, Maryland).
25   This WRAIR formalinized toxoid preparation designated
Tox-F was non-toxic in rabbits at 0.5 mg/kg, the dose at
which SEB toxin is invariably lethal. Furthermore, it
was non-toxic in the murine D-galactosamine model of SEB
toxicity even at 500 μg per BALB/C mouse; 50 μg of SEB is
30   100% lethal in such mice. The physical characteristics
of Tox-F were similar to that described by Eldridge
(Eldridge, J.H., Staas, J.K., Meulbroek, J.A., Tice, T.T.
and Gilley, R.M. Biodegradable and biocompatible poly(DL-
lactide-co-glycolide) microspheres as an adjuvant for
35   staphylococcal enterotoxin B toxoid which enhances the
level of toxin-neutralizing antibodies. Infect. lmmun.

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59: 2978-2986, 1991) is that SDS-PAGE gel of Tox-F showed
two distinct bands with estimated MW of 23,000 and
46,000. Biologically, Tox-F also had the characteristics
previously reported by Eldridge et al., namely in a Mouse
5   Spleen Lymphocyte Proliferative Assay in which
concentrations of SEB toxin of 0.37-10.0 μg/ml were
mitogenic, Tox-F was entirely non-mitogenic at all
concentrations tested (0.04-1 00.0 μg/ml).

10 *Preparation of SEB- Toxoid F*
Formalinized SEB-Toxoid (Tox-F) was prepared according to
the method of Warren, J.R., Spero, L. and Metzger, J.F.
1983. J. lmmunol. 111: 885-892 and as per Eldridge, J.H.
et al. 1991, Infect. Immun. 59: 2978-2986 by formalin
15 treatment for 30 days at 37°C, pH 7.5.

Preparation of SME

Preparation of oil phase
20 Oil phase was composed of MCT oil (0.77g, Mygliol 812,
Hulls, Germany), lecithin (0.14 g, Lipoid E-80, Germany)
and DL-α-tocopherol succinate (9.0 mg, Merck, Germany).
The lipids and oil were weighed in a 250-ml beaker and
mixed at room temperature using a magnetic stirrer during
25 2-4 hours until a homogenous and almost clear solution
was obtained.

Preparation of water phase
Polysorbate 80 (0.5% w/v, Montanox 80, DF, Seppic,
30 France), Glycerol (2.2% w/v, Merck, Germany), EDTA (0.1%
w/v, Merck, Germany), and purified water (to 100% w/v)
were dissolved at room temperature in a 250-ml beaker by
gently shaking using a magnetic stirrer plate until a
clear homogenous solution was obtained (about 15-20 min).
35 A total volume of 45 ml of water phase was prepared. A
vial containing 10 ml of SEB-Toxoid F in 6.3 ml buffer

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was added to the water phase and the mixture was gently
shaken for 5 min.

Preparation of oil-in-water coarse emulsion
5   An oil-in-water emulsion containing the antigen was
prepared by heating the oil phase to 40°C and mixing it
with the water phase with the aid of a 10 ml glass
pipette until a homogenous and milky dispersion was
obtained. The resultant micronsize emulsion was cooled
10   at room temperature.

Sizing of emulsion to submicron range
The droplet size of the emulsion obtained after the
Polytron step was lowered to submicron (nanosize) range
15   by subjecting the emulsion to high shear homogenization
using the Gaulin Microlab 70 High Pressure Homogenizer
(APV Gaulin, Germany) at 800 bar pressure. A total of 5
cycles were performed. The particle size distribution of
the resultant formulation was determined using an N4MD
20   Coulter Particle Size Analyzer (Coulter Electronics,
England). The differential weight % mode of the
instrument indicated the existence of a single and
homogenous population of SME droplets with a mean
particle size distribution of 47.5±58 nm (see Fig. 1).
25   The estimated final antigen concentration in the
formulation was 220 μg/ml.

Example 6:   Preparation of Extrinsic SME vaccine
30   **containing Staphylococcus Enterotoxin B**
     Toxoid-F
Extrinsic formulations of SEB-Toxoid-F in SME were
performed by preparing plain SME as described in Example
5, but in the absence of the antigen and adding
externally the aqueous solution containing the SEB-
35   Toxoid-F to the plain SME by gently shaking for 15 min at
room temperature. A total volume of 0.780 ml of stock

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SME were mixed with 0.780 ml solution of SEB-Toxoid-Fin
0.01 M Tris 0.15M NaCl buffer containing 0.780 mg
protein to give a final SEB-Toxoid-F concentration of
0.500 mg/ml.

5

**Example 7:   Preparation of Extrinsic SME vaccine
containing Staphylococcus Enterotoxin B
Toxoid F complexed to proteosomes**

Extrinsic formulations of SEB-Toxoid-F in SME were
performed by preparing plain SME as described in Example
10   5, but in the absence of the antigen and adding
externally the aqueous solution containing the SEB-
Toxoid-F complexed to proteosomes to the plain SME by
gently shaking for 15 min at room temperature. A total
volume of 0.780 ml of stock SME were mixed with 0.780 ml
15   solution of SEB-Toxoid-F complexed to proteosomes in 0.01
M Tris 0.15M NaCl buffer containing 0.780 mg protein to
give a final SEB-Toxoid-F concentration of 0.500 mg/ml.

**Example 8:   Preparation of Extrinsic-SME vaccine
20   containing Staphylococcus Enterotoxin B
Toxoid C**

Preparation of SEB-Toxoid-C
Carboxymethylated Toxoid (Tox-C): Carboxymethylated with
0.4 Bromoacetic Acid, pH 7.0 for 11 or 21 days in the
25   dark at 20°C as per Stema, G.N. and Bergdoll, M.S. 1982.
Biophys. Biochem. Res. Commun. 105: 121-126 and as per
Scheuber *et al*. 1985. Infect. lmmunol. 50: 869-876.
Made at WRAIR according to described methods.

30   Extrinsic formulations of SEB-Toxoid-C in SME were
performed by preparing plain SME as described in Example
5, but in the absence of the antigen and adding
externally the aqueous solution containing the SEB-
Toxoid-C to the plain SME by gently shaking for 15 min at
35   room temperature. A total volume of 0.165 ml of stock
SME were mixed with 0.110 ml solution of SEB-Toxoid-C in

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water containing 0.110 mg protein to give a final SEB-
Toxoid-C concentration of 0.400 mg/ml.

5   Example 9:   Preparation of Extrinsic SME vaccine
                   containing Staphylococcus Enterotoxin B
                   Toxoid-C complexed to proteosomes

Extrinsic formulations of SEB-Toxoid-C in SME were
performed by preparing plain SME as described in Example
5, but in the absence of the antigen and adding
externally the aqueous solution containing the SEB-
10   Toxoid-C complexed to proteosomes to the plain SME by
gentle shaking for 15 min at room temperature. A total
volume of 0.045 ml of stock SME were mixed with 0.230 ml
solution of SEB-Toxoid-C complexed to proteosomes in
water containing 0.110 mg protein to give a final SEB-
15   Toxoid-C concentration of 0.400 mg/ml.

     Example 10:   Preparation of Mucoadhesive Extrinsic SME
                    Formulation containing 0.05% Carbopol

420 ml of distilled water in which were dissolved 0.250 g
20   Carbopol-940 (Goodrich, U.S.) and 11.2 g glycerol
(isotonic agent), pH 3.82, were warmed to 45°C and mixed
with the oil phase, consisting of 21.2 g MCT oil (medium
chain triglycerides, SIO, France), 3.74 g Lipoid E-75
(egg lecithin) and 1.5% w/v Emulfor EL-620 (Rhone-
25   Poulenc, France), at a temperature of 60°C. After mixing
by high speed stirrer (Polytron 3000, Kinematica,
Switzerland) at 20,000 rpm for 5 minutes the mixture was
dispersed by a high pressure homogenizer (Gaulin Microlab
70) at 700 bar for 5 minutes (approximately 10 cycles).
30   The resulting emulsion was cooled, and after adjusting
the pH to 5.0, the emulsion was filtered and packed in
sterile bottles through a 0.2 μm filter.

     After filtering, droplet size was measured by the photon
35   correlation spectroscopy using a particle size analyzer
(N4MD, Coulter Electronics, U.S.A.). The droplet size for

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carbopol containing droplets was 127±79 nm. This
mucoadhesive composition was prepared as an extrinsic
plain SME formulation to be added to the specific
immunogen by gentle mixing.

5

Example 11:   Enhanced Immunogencity to SEB Antigen
                after Parenteral Immunization with
                Intrinsic and Extrinsic SEB-Toxoid F-SME
                Vaccines compared to Free Antigen or Alum-
                Adjuvanted Vaccine

10

The antigen used was Staphylococcal Enterotoxin B (SEB)
toxoid F alone or SEB toxoid complexed to meningococcal
outer membrane proteosomes. This antigen was formulated
with SME adjuvant either intrinsically or extrinsically,
15 as described in Examples 5, 6 and 7, and compared to SEB
toxoid-F alone or adjuvanted with alum. Outbred CD-1
mice, 5 animals/group, were immunized twice at
approximately 3 week intervals by intramuscular
injections with 10 or 50 µg doses of SEB Toxoid F. Sera,
20 obtained after first and second immunizations, were
analyzed by ELISA techniques using anti-SEB as the
detecting antibody. As shown in Figures 2 and 3,
intrinsic and extrinsic-SME formulations with (Fig. 2) or
without proteosomes (Fig. 3) were effective in enhancing
25 immunity to SEB antigens. In all the cases, the anti-SEB
serum IgG titers obtained with the SME-adjuvant were
several orders of magnitude higher than those obtained
with the alum-adjuvanted formulation.

30 Example 12:   Enhanced lmmunogenicity to SEB Antigen
                after Parenteral Immunization with SEB-
                Toxoid-C SME Vaccines compared to Free
                Antigen or Alum-Adjuvanted Vaccine.

The antigen used was carboxymethylated SEB-Toxoid or
Toxoid-C. The antigen was incorporated extrinsically in
35 SME, as described in Example 9. The immunization
protocol and sera analysis for antibody activity was as

- 30 -

described in Example 11. Mice were immunized
intramuscularly with two 10μg doses of Toxoid-C. As
shown in Table 1, parenteral immunization with Toxoid-C-
SME vaccine induced a 4-fold increase in the level of
5 serum IgG even in the absence of proteosomes.

TABLE 1. Effects of formulation with saline, alum or
submicron emulsion (SME) on parenteral (intramuscular)
immunogenicity of carboxymethylated SEB toxoid (Toxoid-C)

Antigen (2 doses)	Adjuvant	Anti-SEB serum IgG titer (OD>0.5 at 1 hr)
SEB Toxoid-C	None (saline)	819,200
SEB Toxoid-C	Alum	6,553,600
SEB Toxoid-C	SME	3,276,800

Example 13:   **Intranasal Immunization with SEB-Toxoid F
SME Vaccines**

Immunization against biologic threat agents, such as SEB
20 requires the development of vaccines that can protect
against respiratory challenge. The induction of
respiratory IgA as well as serum IgG is likely to be
critically important to the success of such vaccines. To
elicit effective respiratory, as well as systemic,
25 immunity it may be necessary to develop a vaccine system
that can be delivered intranasally, as well as
parenterally.

BALB/c mice (8 animals/group) were immunized twice by
30 intranasal slow dropwise instillation into both nostrils
with SEB-Toxoid F (10 μg doses) formulated with or
without proteosomes as a free antigen or adjuvanted with
SME. Immunogenicity of these formulations was evaluated
after the two immunizations by determining systemic IgG
35 antibody activity against SEB and induction of anti-SEB
respiratory IgA antibody titers.

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As shown in Table 2, Toxoid-F formulated in extrinsic SME
either with or without proteosomes conferred intranasal
immunogenicity resulting in 500- to 2000-fold enhancement
of anti-SEB serum IgG antibody activity.

TABLE 2. Effects of formulation of formalized SEB
(Toxoid-F) with submicron emulsion (SME) and/or
proteosomes on induction of anti-SEB serum IgG following
intranasal immunization

Antigen (2 doses)	Adjuvant	Anti-SEB serum IgG titers (O.D.>0.5 at 1 hr)	
		w/o protoeosomes	formulated with proteosomes
SEB Toxoid-F	None (saline)	100	51,200
SEB Toxoid-F	SME	51,200	204,800

Extrinsic SME formation of Toxoid-F complexed to
proteosomes also enhanced lung IgA antibody levels to SEB
(Table 3).

TABLE 3. Enhanced production of anti-seb respiratory
(lung) IgA antibodies after intranasal immunization of
BALB/c mice with 10 μg SEB-Toxoid F antigen alone or
adjuvanted with SME

Antigen	Adjuvant	Anti-SEB lung IgA titer
SEBtox-F	None (saline)	0
SEBtox-F	Extrinsic SME	0
SEBtox-F	Intrinsic SME	0
SEBtox-F -proteosomes	None (saline)	0.6
SEBtox-F -proteosomes	Extrinsic SME	10

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Example 14:   Intranasal immunization with
                  lipopolysaccharide (LPS) antigen from
                  S.flex complexed to proteosomes adjuvanted
                  with Extrinsic-SME.

Evidence that the SME has potential as a mucosal adjuvant
5   is also shown by the ability of the extrinsic-SME
adjuvant formulation to markedly enhance the lung IgA of
the proteosome-LPS antigen when given intranasally (Table
4) compared to the free antigen. The immunization
protocol included two groups of BALB/c mice (4 animals
10 each) which were immunized intranasally with 10 μg does
of LPS from S.flex compared to proteosomes. The non-
covalent complexation of LPS to proteosomes was carried
out in a similar way as described in Example 2.

15 TABLE 4. Induction of mucosal immunity after intranasal
immunization of Balb/C mice with 10 μg LPS-complexed to
proteosomes as a free antigen or adjuvanted with
extrinsic-SME.

Antigen	Adjuvant	Anti-S.flex Lung LPS IgA			
LPS (S.flex)- Proteosomes	None (saline)	256	512	1024	2
LPS (S.flex)- Proteosomes	Extrinsic SME	>2048	>2048	>2048	>2048

25

Example 15: Oral (intragastric) immunization of mice
with lipopolysaccharide (LPS) antigen from S.flex
complexed to proteosomes adjuvanted with extrinsic-SME.
BALB/c mice (5 animals/group) were immunized orally
(intragastrically) with 100 μg does of LPS complexed to
30 proteosomes and adjuvanted with extrinsic SME. As shown
in Table 5, the SME adjuvant formulation was able to
induce higher anti-S.flex LPS intestinal IgA titers,
compared to the free antigen.

35

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TABLE 5.   Induction of mucosal immunity after oral
(intragastric) immunization of Balb/C mice with LPS-
complexed to proteosomes as a free antigen or adjuvanted
with extrinsic-SME.

Antigen	Adjuvant	Anti S.flex LPS intestinal IgA
LPS (S.flex)- Proteosomes	None	74
LPS (S.flex)- Proteosomes	Extrinsic- SME	169

Example 16:   Protection against systemic challenge with
               SEB by SEB Toxoid vaccines formulated with
               SME adjuvant administered parenterally or
               intranasally

Mice immunized parenterally (Table 6) or intranasally
(Table 7) with Staphylococcus Enterotoxin B in mice
immunized with SEB Toxoid-F vaccines formulated with SME
or proteosomes were significantly protected against
systemic SEB challenge (100 µg toxin).

TABLE 6.   Correlation of anti-SEB serum IgG titers
obtained after parenteral (i.m.) immunization (50 µg SEB-
Toxoid F) with protection against systemic challenge with
100 µg SEB ion CD-1 mice.

Antigen	Adjuvant	Anti-SEB IgG	died/total	Survival
control	none	0	10/18	44%
SEB-tox F	none	3,200	3/5	40%
SEB-tox F	alum	51,200	4/5	20%
SEB-tox F	SME- Extrinsic	204,800	0/5	100%
SEB-tox F	SME- Intrinsic	204,800	0/4	100%

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TABLE 7. Correlation of anti-SEB serum IgG titers with
protection against systemic (im) challenge with 100 μg
SEB in D-galactosamine-sensitized inbred (Balb/C) mice:
effect of proteosomes and SME on efficacy of intranasal
immunization with formalinized (Toxoid-F) SEB vaccines.

Vaccine	Adjuvant	Anti-SEB IgG serum titer	SEB challenge results	
			#died/ #total	Survived
None	None	<50-	12/12	0%
Toxoid-F	SME	51,200	4/7	40%
Toxoid F-Proteosomes	None	409,600	2/5	60%

The data in Table 6 show a very good correlation between
the anti-SEB serum IgG titers obtained after
intramuscular immunization of CD-1 mice with protection
against systemic challenge with 100 μg of SEB. In the
groups immunized with either extrinsic or intrinsic SME-
SEB Toxoid F vaccines, the survival was 100% while for
animals immunized with free antigen or alum-adjuvanted
vaccine the survival was 0 and 40%, respectively.

Table 7 shows similar data for BALB/c mice immunized
intranasally with SEB-Toxoid F alone or complexed to
proteosomes in SME adjuvant or as free antigen.

Example 17:   Enhanced murine immunogenicity of gp 160
                 HIV antigen incorporated in SME adjuvants
                 either intrinsically or extrinsically.

The antigens used were gp 160 alone or gp 160 complexed
to meningococcal outer membrane proteosomes. These
antigens were formulated with SME either intrinsically or
extrinsically and compared to the gp 160 used without an
adjuvant, as described in Examples 1-4. Mice (5
animals/group) were immunized three times at 3-4 week
intervals. Sera, obtained after 2 and 3 immunizations

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were analyzed for specific anti-gp 160 peptide IgG
responses by quantitative Western blot techniques using
seven specific HIV epitopes as the detecting antigens.
As shown in Table 8a, both intrinsic and extrinsic
5   formulations of SME were effective in enhancing immunity
to several gp160 epitopes. Note that the strongest and
broadest anti-HIV responses were obtained with the
intrinsic formulation using gp 160-proteosomes as the
antigen (rows 5 and 11 in Table 8a).
10

Example 18:   Enhanced lapine immunogenicity of gp160
HIV antigen free or complexed to
proteosomes formulated in SME vaccine
adjuvants.

The antigens used were gp160 alone or gp160 complexed to
15   meningococcal outer membrane proteosomes. These antigens
were formulated with SME either intrinsically or
extrinsically and compared to gp160-alum, gp160-
proteosomes, and gp160-proteosomes-alum formulations.
Rabbits (4 animals/group) were immunized parenterally
20   four times at different intervals. Sera, obtained after
second, third and fourth immunizations were analyzed by
for specific anti-gp160 peptide IgG responses by
quantitative Eastern blot techniques using seven specific
HIV epitopes as the detecting antigens. As shown in
25   Table 8b, intrinsic formulations with SME with or without
proteosomes were effective in enhancing immunity to
several gp160 epitopes and even gp160 formulated with
only an SEM-intrinsic preparation was as good as or
better than gp160 formulated with alum, a known adjuvant
30   currently used in people.

35

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TABLE 8a: Sub-Micron Emulsions Enhance the Immunogenicity of Proteins alone or Protein-Proteosomes Complexes in Mice

Antigen	Adjuvant	EPITOPES						
		C1 48-128	C21E 254-274	C3 342-405	CKen 735-752	C41 579-605	C448 453-518	V3 290-338
		Conserved areas of gp120			gp40 areas of gp160			variable loop
After 2 Immunizations								
gp160	none	1	157,447	1	1	73,089	397,458	32,979
gp160	SME-intrinsic	131,848	36,805	1	1	39,357	123,000	1
gp160	SME-extrinsic	111,102	215,056	1	88,089	76,348	596,159	1
gp160-proteosomes	none	325,008	870,970	1	48,437	65,787	994,290	17,758
gp160-proteosomes	SME-intrinsic	1,772,367	1,237,152	162,326	1	161,763	411,147	1
gp160-proteosomes	SME-extrinsic	678,516	254,246	17,205	1	224,494	566,667	18,260
After 3 Immunizations								
gp160	none	354,732	350,071	1	13,879	183,047	535,709	62,257
gp160	SME-intrinsic	131,211	186,281	1	2,807	119,449	130,965	1
gp160	SME-extrinsic	241,500	382,106	1	56,308	105,334	551,054	46,416
gp160-proteosomes	none	576,316	617,847	1	149,657	346,582	922,360	416,493
gp160-proteosomes	SME-intrinsic	2,423,815	1,201,061	196,191	6,196	310,867	873,932	61,281
gp160-proteosomes	SME-extrinsic	88,454	584,250	4,298	19,432	64,524	301,775	132,800

TABLE 8b: Sub-Micron Emulsions Enhance the immunogenicity of Proteins alone in Rabbits

Data From Rabbit Immunogenicity

After 2 Immunizations								
gp160	alum	92,402	63,382	3,288	35,913	29,721	22,150	10,183
gp160	SME-intrinsic	95,403	84,672	27,840	50,221	103,119	94,622	88,896
gp160	SME-extrinsic	126,521	143,598	6,243	2,604	86,541	69,847	44,426
After 3 Immunizations								
gp160-proteosomes	alum	56,202	78,323	4,281	17,380	24,368	7,320	7,623
gp160-proteosomes	SME-intrinsic	36,980	13,608	5,339	6,333	21,318	36,369	54,347
gp160-proteosomes	SME-extrinsic	111,852	90,717	12,986	10,229	77,818	68,167	54,176

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Another analysis of the experiment showing adjuvant
effects of SME with gp160 vaccines in rabbits in which
the antigens used were gp160 alone or gp160 complexed to
meningococcal outer membrane proteosomes is shown in
5   Figures 4 and 5. These antigens were formulated with SME
either intrinsically or extrinsically and compared to
gp160-alum, gp160-proteosomes, and gp160-proteosomes-alum
formulations. Rabbits (4 animals per groups) were
immunized parenterally four times at different intervals.
10 Sera, obtained after second, third and fourth
immunizations were analyzed by ELISA techniques using
several specific HIV epitopes as the detecting antigens.
As shown in Figure 4, intrinsic formulations with SME
with or without proteosomes were effective in enhancing
15 immunity to several gp160 epitopes.

Among the six groups tested, the intrinsic-SME vaccine
formulation containing the gp160 antigen in the absence
of alum, was the most effective vaccine eliciting the
20 high antibody titers with improved and extended
immunogenicity against several selected gp 160 epitopes
compared to the standard alum-adjuvanted vaccine actually
in clinical trials.

25 More importantly, the SME-intrinsic formulations of gp160
were able to induce IgG antibodies against the V3 loop
epitope of the gp160 molecule (Fig. 5 and Table 8b), the
site of the principal neutralizing determinant which
blocks binding to CD4 (the main cellular receptor for
30 HIV). Since the standard alum-adjuvanted formulation did
not generate antibodies against the V3 peptide domain,
the additional epitope recognition and enhanced total
immunogenicity of SME-intrinsic adjuvant is considered to
be a very significant achievement.
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These data are encouraging for the development of
enhanced subunit formulations of gp160 vaccines for HIV
vaccine therapy.

5   **Example 19:**   **Immunogenicity of *Leishmania* glycoproteins**
                       formulated in extrinsic-SME adjuvant
                       either with or without proteosomes.

The gene for a surface protein antigen of *Leishmania*
major gp63, has been cloned and sequenced. This protein,
recombinantly expressed in live *Salmonella,* or given in a
10 sub-unit vaccine as either the purified native gp63 or
selected gp63 peptides (Jardim A., Alexander J., Teh S.,
Ou D, Olafson R.W. 1990. J. Exp. Med. 172: 645), has
recently been shown to limit the extent to lesion
development in murein models of cutaneous leishmaniasis
15 when given with certain adjuvants. These results suggest
that a vaccine to protect humans against leishmaniasis
composed of defined purified components is a realistic
goal. The sub-unit vaccines were effective, however,
only when administered with adjuvants containing
20 *Corynebacterium parvum* (CPV) and poloxamer 407. Other
adjuvants (Complete Freund's Adjuvant, CFA), or lack of
adjuvant exacerbated disease.

Major success was achieved with the discovery that
25 subcutaneous immunization with one small gp63 peptide
covalently conjugated to lauryl-cysteine protected
against sever *Leishmania* cutaneous lesions with reduction
of lesions in three separate experiments.

30 The objective in the present example was to demonstrate
immunogenicity and efficacy of an SME-adjuvanted
lipopeptide vaccine to protect against severe morbidity
of cutaneous leishmaniasis in murein models.

35

TABLE 9.   Effect of immunization with LC-467 *Leishmania*
lipopeptide formulated in SME adjuvant either with or
without proteosomes on lesion size.

Formulation	% decrease on lesion size from Control in CBA
LC-467-proteosomes	52
LC-467-Extrinsic SME	90
LC-467-proteosomes-Extrinsic SME	90

The antigen used were lipopeptides obtained from the
major glycoprotein of the *Leishmania* parasite.   The
peptides (denoted 457 and 154) were covalently attached
to lauryl cysteine to serve as the hydrophobic foot.   The
vaccine was prepared by gentle mixing of the antigens at
room temperature with plain extrinsic-SME for 15 min.

Two animal models were used: (1) CBA mouse, model similar
to human cutaneous disease; and (2) BALB/c mice, model
similar to visceral *Leishmania* which is lethal if not
treated.

The immunization protocol included two injections of the
animals (8 mice/group) at weeks 0 and 3 with the
experimental vaccines (50 μg peptide/mouse).   At week 6
the mice were infected with live *Leishmania* parasites and
the lesion size as function of time was measured and
compared.   The results were expressed as % decrease from
control (PBS injection).   Different vaccine formulations
containing SME with or without proteosomes were tested.
Appropriate control formulations were used.

All the formulations tested containing the 154
glycopeptide had no effect on lesion size, even if this
vaccine contained proteosomes, SME or their combination.
However, when the LC-467-glycopeptide was used, up to 90%

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decrease in lesion size was obtained by incorporation of the lipopeptide in SME, even in the absence of proteosomes (Table 8).

Since there is considerable homology among
5   *Leishmania* strains, this peptide may have wide application in ameliorating lesions caused by other forms of *Leishmania*.

Incorporation by reference
10   To the extent necessary to understand or complete the disclosure of the present invention, all publications, patents, and patent applications mentioned herein are expressly incorporated by reference therein to the same extent as though each were individually so
15   incorporated.

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THE CLAIMS

What is claimed is:

    1.   A vaccine adjuvant composition of an oil-in-water submicron emulsion consisting essentially of about
5 0.5 to 50% of a first component of an oil, about 0.1 to 10% of a second component of an emulsifier, about 0.05 to 5% of a non-ionic surfactant, about 0.00001 to 1% of an immunogen, and an aqueous continuous phase, said submicron emulsion having a mean droplet size in the
10 range of between about 0.03 and 0.5 μm.

    2.   The emulsion of claim 1 wherein the mean droplet size is between about 0.05 and 0.2 μm.

15   3.   The emulsion of claim 1 wherein the first component is a medium chain triglyceride oil, a vegetable oil, or mixtures thereof.

    4.   The emulsion of claim 3 wherein the first
20 component is present in an amount of about 1 to 20%.

    5.   The emulsion of claim 1 wherein the emulsifier is a phospholipid compound or a mixture of phospholipids.

25   6.   The emulsion of claim 5 wherein said emulsifier is selected from the group consisting of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, phosphatidic acid, sphingomyelin
30 and cardiolipin.

    7.   The emulsion of claim 6 wherein the emulsifier is present in an amount of about 0.2 to 5%.

35   8.   The emulsion of claim 1 wherein the surfactant is selected from the group consisting of fatty alcohols,

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polyethylene glycol esters of fatty acids,
polyethoxylated fatty acid esters, polyethoxylated fatty
alcohol ethers, polyethoxylated alkylphenyl ethers.

5   9.   The emulsion of claim 1 wherein the surfactant
is an alkylene oxide condensate of an organic compound
having one or more hydroxyl groups.

       10.   The emulsion of claim 9 wherein the non-ionic
10 surfactant is present in an amount of about 0.1 to 5%.

       11.   The emulsion of claim 1 wherein the first
component is present in an amount of about 1 to 20%, and
the second component and the non-ionic surfactant are
15 each present in an amount of about 0.1 to 2%.

       12.   The composition of claim 1 wherein the
immunogen is hydrophilic, lipophilic or amphiphilic.

20   13.   The composition of claim 1 wherein the
immunogen is a native, recombinant or synthetic peptide,
protein or glycoprotein derived from a bacteria, virus or
parasite.

25   14.   The composition of claim 13 wherein the antigen
is the gp160 envelope protein of the HIV virus, or a
fragment thereof.

       15.   The composition of claim 13 wherein the antigen
30 is the surface glycoprotein of a *Leishmania* parasite or a
fragment thereof.

       16.   The composition of claim 15 wherein the surface
glycoprotein or peptide is covalently conjugated to a
35 hydrophobic foot.

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17.   The composition of claim 16 wherein the hydrophobic foot is lauryl-cysteine.

18.   The composition of claim 1 wherein the immunogen is a protein toxoid.

19.   The composition of claim 18 wherein the immunogen is Staphylococcus Enterotoxin B toxoid.

20.   The composition of claim 1 wherein The immunogen is an oligosaccharide, a polysaccharide or a lipopolysaccharide.

21.   The composition of claim 20 wherein the immunogen is a lipopolysaccharide from *Shigella flexneri*.

22.   The composition of claim 1 wherein the immunogen is complexed to proteosomes.

23.   The composition of claim 1 which is essentially free of added amounts of muramyl peptides or their lipophilic derivatives.

24.   A vaccine adjuvant composition of an oil-in-water submicron emulsion consisting essentially of about 0.5 to 50% of a first component of an oil, about 0.1 to 10% of a second component of an emulsifier, about 0.05 to 5% of a non-ionic surfactant, about 0.00001 to 1% of an immunogen, and an aqueous continuous phase, said submicron emulsion having a mean droplet size in the range of between about 0.03 and 0.5 μm, in combination with a bioadhesive or mucoadhesive macromolecule in an

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amount sufficient to impart adhesive properties to the vaccine.

25. The composition of claim 24 wherein the
5 mucoadhesive macromolecule is selected from the group consisting of acidic naturally occurring polymers; acidic synthetically modified natural polymers; acidic nonnatural polymers; basic polymers; neutral nonnatural polymers; and neutral naturally occurring polymers.
10

26. The composition of claim 25 wherein the acidic nonnatural polymer is selected from the group consisting of polymers and copolymers containing acrylic acid units, polymers and copolymers containing methacrylic acid
15 units, and poly(methylvinylether/maleic anhydride) copolymers.

27. The composition of claim 25 wherein the acidic nonnatural polymer is polyacrylic acid.
20

28. A vaccine adjuvant composition of an oil-in-water submicron emulsion consisting essentially of about 0.5 to 50% of a first component of an oil, about 0.1 to 10% of a second component of an emulsifier, about 0.05 to
25 5% of a non-ionic surfactant, about 0.00001 to 1% of an immunogen, a cryoprotectant in an amount sufficient to facilitate lyophilization of the composition, and an aqueous continuous phase, said submicron emulsion having a mean droplet size in the range of between about 0.03
30 and 0.5 μm.

29. The composition of claim 28 wherein the cryoprotectant is selected from the group consisting of an amino acid, an oligopeptide and polyvinylpyrrolidone.
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30. A dehydrated vaccine adjuvant composition
obtained by lyophilizing the composition of claim 28 or
29, whereby a submicron emulsion is generated when water
is added to the dehydrated composition.

5

31. A dehydrated vaccine adjuvant composition for
administration of an immunogen, comprising a plurality of
submicron particles having a hydrophobic core, the
immunogen, and a surfactant, whereby a submicron emulsion
10   is generated when water is added to the dehydrated
composition.

32. A method for preparing the composition of claim
1 by an intrinsic procedure, which comprises adding the
15   immunogen to the oil phase before emulsification of the
water and oil phases.

33. A method for preparing the composition of claim
1 by an intrinsic procedure, which comprises adding the
20   immunogen to the water phase before emulsification of
water and oil phases.

34. A method for preparing the composition of claim
1 by an extrinsic procedure, which comprises preparing a
25   submicron emulsion from the oil, surfactant and
emulsifier components, and adding the immunogen
externally by mixing it with the previously prepared
submicron emulsion.

30   35. A vaccine adjuvant composition prepared by
adding water to the dehydrated composition of claim 31 to
generate a submicron emulsion which contains the
immunogen therein.

35   36. A method for providing enhanced immunogenicity
in a subject which comprises administering the

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composition of claim 1, 24, 28 or 35 to the subject to
introduce the immunogen therein.

37.   The method of claim 36 which further comprises
administering the composition parenterally, orally,
intranasally or topically to the subject.

38.   A method of achieving mucosal immunity in a
subject which comprises administering the composition of
claims 24 to 27 to mucosal surfaces of the subject to
introduce the immunogen therein.

39.   The method of claim 38 which further comprises
applying the composition at least upon a portion of the
vaginal, nasal, rectal or oral mucosa of the subject.
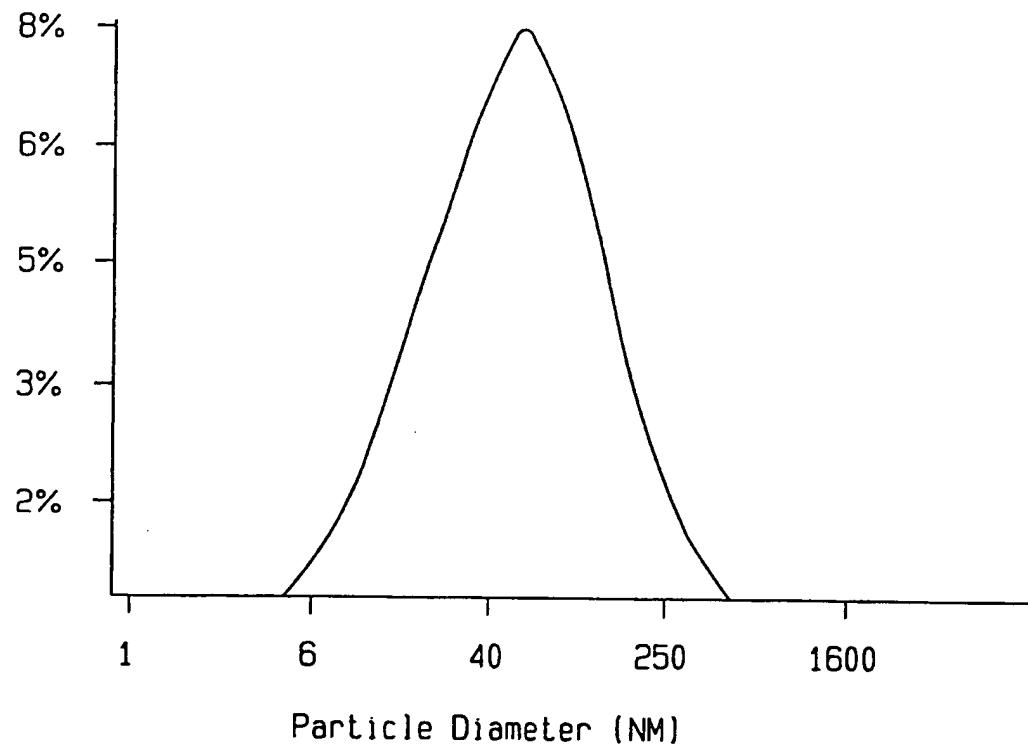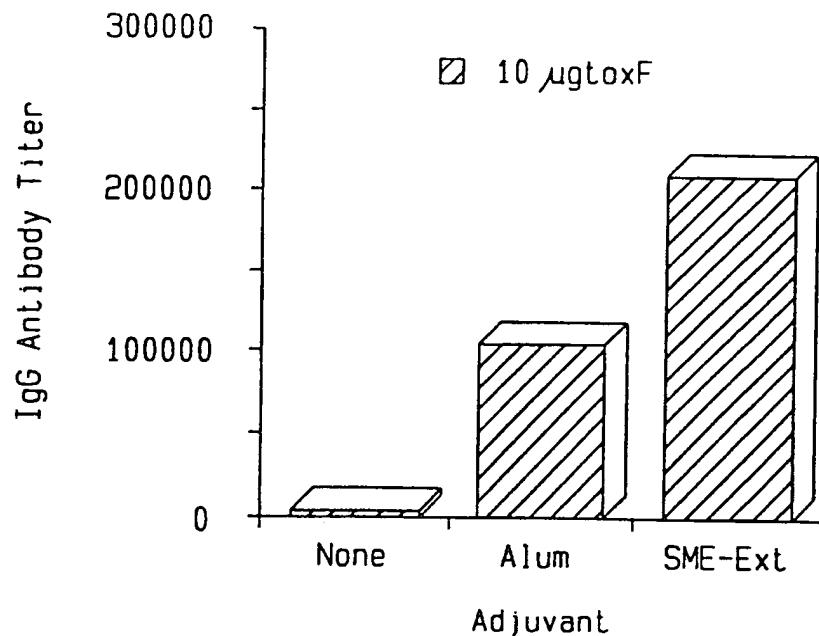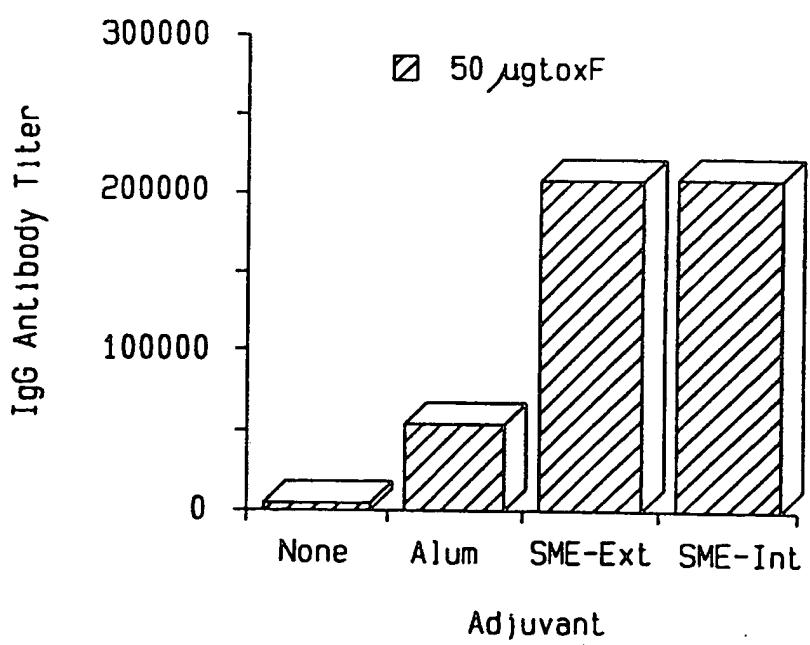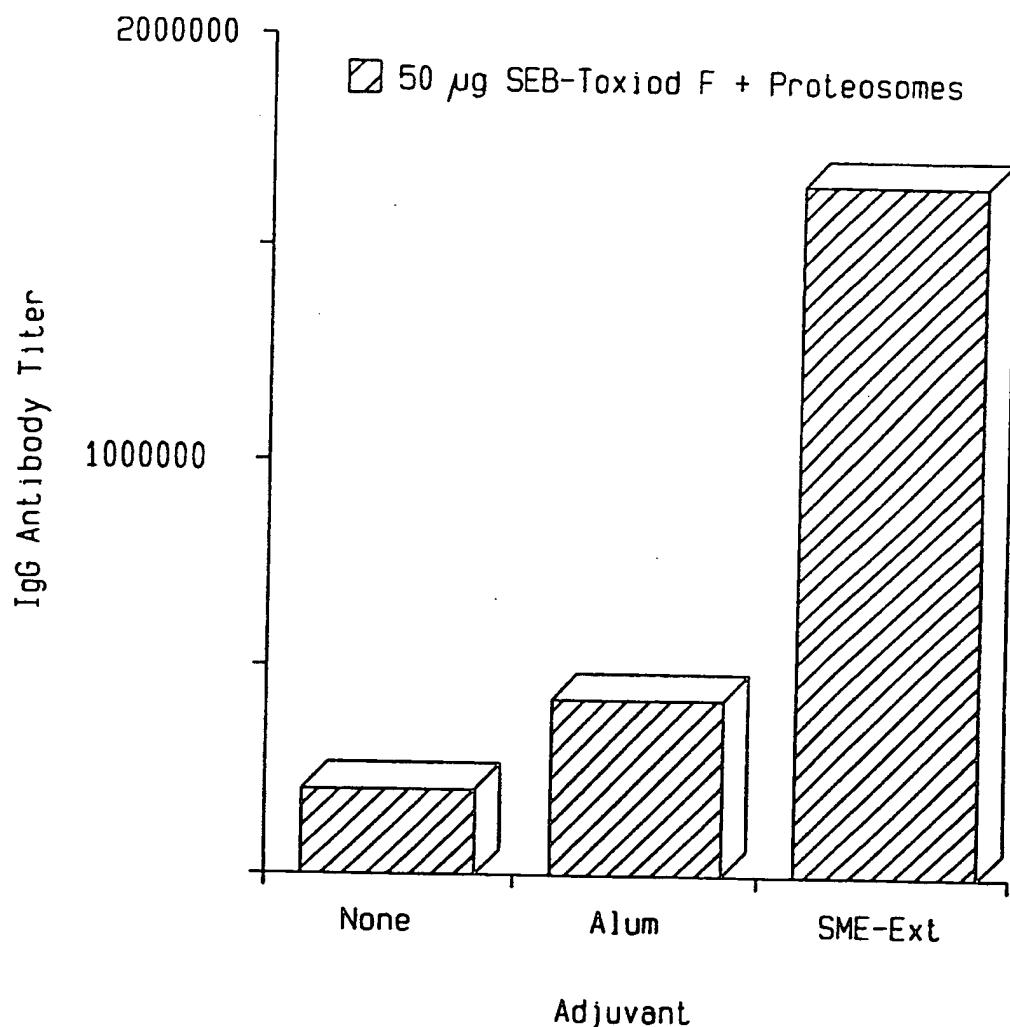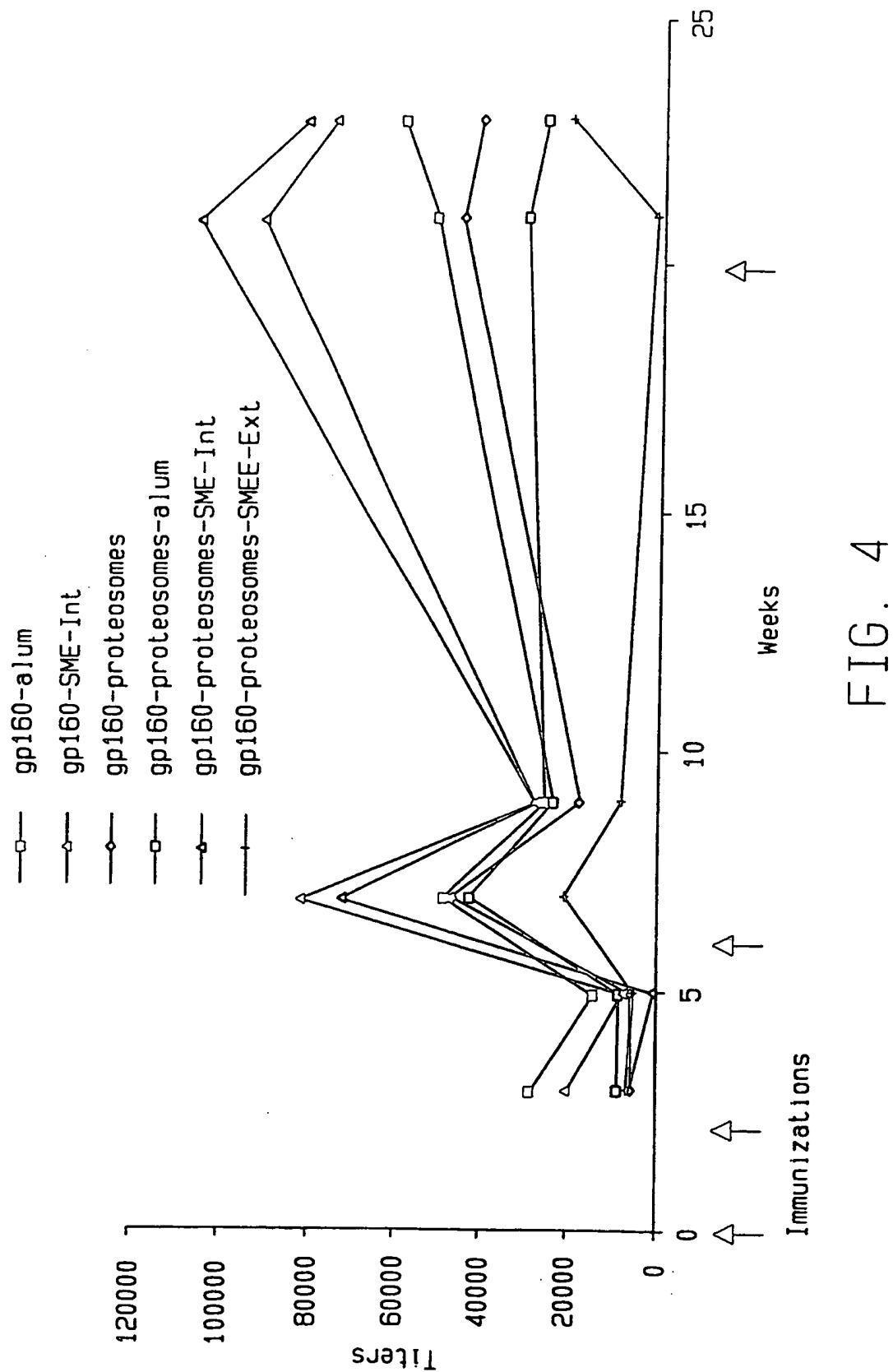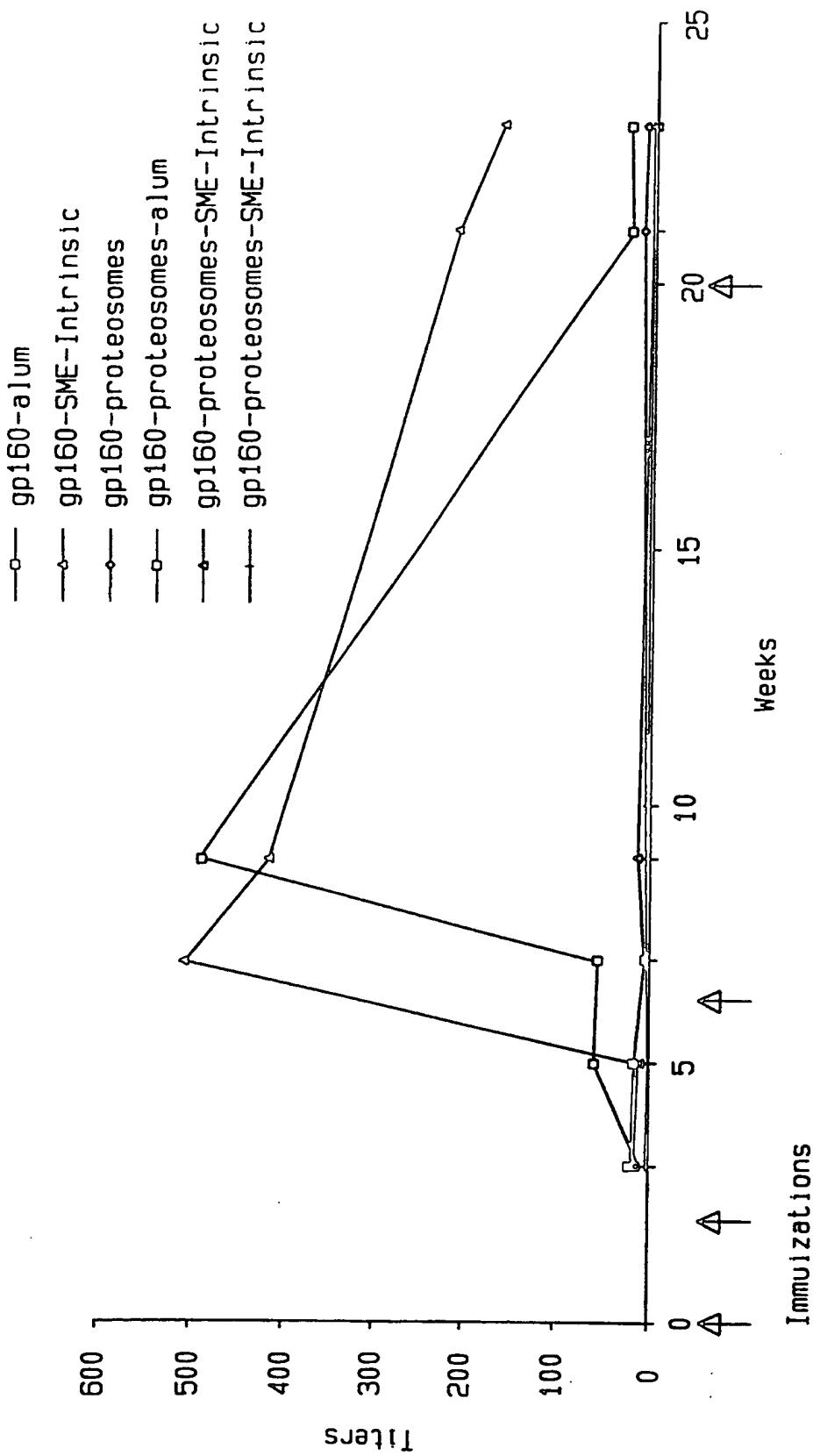
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FIG. 1

FIG. 2A

FIG. 2B

FIG. 3

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FIG. 4

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FIG. 5

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 39/008, 39/085, 39/21, 9/10, 9/107, 9/127, 37/20
US CL :424/88, 89, 452, 450, 462

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/88, 89, 452, 450, 462; 436/829; 514/937, 938

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Medline, Biosis, Embase, Chem Abstracts

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A, 92/00081 (Barchfeld et al) 09 January 1992, see pp. 5-7, 11, 20, 23, 25, 27, 30-33.	1-39
Y	US, A, 5,026,543 (Rijke) 25 June 1991, see column 1, lines 21-29.	1-39
Y	Biotechnology Therapeutics, Volume 2, Nos. 1-2, issued 1991, Barrett et al, "Characterization of a Vaccinia-Derived Recombinant HIV-1 gp160 Candidate Vaccine and its Immunogenicity in Champanzees", pages 91-106, see pages 91-92.	14

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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		"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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Date of the actual completion of the international search	Date of mailing of the international search report
26 JANUARY 1994	FEB 08 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer JULIE KRSEK-STAPLES
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196
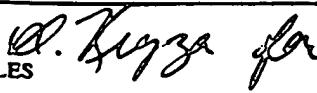
Form PCT/ISA/210 (second sheet)(July 1992)

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Immunology, Volume 72, No. 1, issued January 1991, Yang et al, "Identification and Characterization of Host-Protective T-Cell Epitopes of a Major Surface Glycoprotein (gp63) from *Leishmania major*", pages 3-9, see entire document.	15-17
Y	Journal of Immunology, Volume 140, No. 4, issued 15 February 1988, Russell et al, "Effective Immunization against Cutaneous Leishmaniasis with Defined Membrane Antigens Reconstituted into Liposomes",pages 1274-1279, see entire document.	15-17
Y	Journal of Experimental Medicine, Volume 167, No. 2, issued February 1988, Lowell et al, "Peptides Bound to Proteosomes via Hydrophobic Feet Become Highly Immunogenic Without Adjuvants", page 658-653, see pages 658-659.	16-17
Y	Infection and Immunity, Volume 59, No. 9, issued September 1991, Eldridge et al, "Biodegradable and Biocompatible Poly(DL-Lactide-Co-Glycolide) Micospheres as an Adjuvant for Staphylococcal Enterotoxin B Toxoid Which Enhances the Level of Toxin-Neutralizing Antibodies", pages 2978-2986, see entire document.	18-19
Y	Infection and Immunity, Volume 61, No. 6, issued June 1993, Orr et al, "Immunogenicity and Efficacy of Oral or Intranasal *Shigella flexneri* 2a and *Shigella sonnei* Proteosome-Lipopolysaccharide Vaccines in Animal Models", pages 2390-2395, see entire document.	20-21
Y	Pharmaceutical Research, Volume 9, No. 12, Issued December 1992, Thermes et al, "Mucoadhesion of Copolymers and Mixtures Containing Polyacrylic Acid", pages 1563-1567, see page 1563.	24-27, 38, 39
Y	Journal of Pharmacy and Pharmacology, Volume 37, No. 10, issued October 1985, Morimoto et al, "Enhancement of Nasal Absorption of Insulin and Calcitonin Using Polyacrylic Acid Gel", pages 134-136, see page 134.	24-27, 38, 39
Y	US, A, 3,852,155 (Moore) 03 December 1974, see column 2, lines 9-10.	28-31, 35
Y	US, A, 5,185,146 (Altenburger) 09 February 1993, see column 4, lines 23-33.	28-31, 35
Y	US, A, 4,073,743 (Midler Jr. et al) 14 February 1978, see column 2, lines 51-55.	33

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,795,635 (Peleg et al) 03 January 1989, see column 3, lines 38-42.	34
Y	Analytical Biochemistry, Volume 211, issued 15 May 1993, Friede et al, "Lyophilized Liposomes as Shelf Items for the Preparation of Immunogenic Liposome-Peptide Conjugates", pages 117-122, see page 117.	30, 31, 35
Y	Journal of Immunological Methods, Volume 135, Nos. 1-2, issued 31 December 1990, Ruegg et al, "Preparation of Proteosome-Based Vaccines. Correlation of Immunogenicity with Physical Characteristics", pages 101-109, see page 102.	22